Method for screening of modulators of calcineurin activity

Specification:

The invention relates to a method for screening of modulators of calcineurin.

Calcineurin (E.C. 3.1.3.16) is a serine/threonine phosphoprotein phosphatase and is composed of a catalytic (calcineurin A) and regulatory (calcineurin B) subunit (about 60 and about 18 kDa, respectively). In mammals, three distinct genes (A-alpha, A-beta, A-gamma) for the catalytic subunit have been characterized, each of which can undergo alternative splicing to yield additional variants. Although mRNA for all three genes appears to be expressed in most tissues, two isoforms (A-alpha and A-beta) are most predominant in brain.

Calcineurin has been cloned from various organisms including human (Guerini et al., 1989), (Guerini and Klee, 1989), (Kincaid et al., 1991), (Kuno et al., 1989), (Ito et al., 1989), (Muramatsu and Kincaid, 1993). The crystal structure has shown that calcineurin A contains a binuclear metal center with unknown enzymatic function (Griffith et al., 1995). Recombinant expression of rat calcineurin A subunit in bacteria or SF9-cells were not effective and yielded only poor enzymatic activities since calcineurin A is not stable in the absence of calcineurin B (Perrino et al., 1992), (Perrino et al., 1995), (Haddy and Rusnack, 1994). Coexpression of calcineurin A and calcium binding subunit calcineurin B yielded a more stable and active enzyme (Mondragon et al.,

1997). Calcineurin has been implicated in various neuronal signaling pathways (Klee et al., 1988), (Yakel, 1997) but the neuronal function is only poorly understood (Guerini, 1997).

Calcineurin is the only protein phosphatase known to be under the control of Ca^{2+} and calmodulin. Binding of Ca^{2+} and calmodulin is necessary for enzymatic activity. Calmodulin is bound by the catalytic subunit whereas the regulatory subunit possesses four Ca^{2+} binding sites.

Calcineurin is discussed in the context of immunosuppression. It has been shown that calcineurin acts via the transcription factor NFAT (nuclear factor of activated T cells) on the T cell response. The functions of NFAT proteins are directly controlled by calcineurin in a calcium- and calmodulin-dependent manner. Activation of NFAT by calcineurin is mediated by the cytosolic binding protein FKBP.

Substances which are able to block the calcineurin signal pathway are suitable agents in order to block the T cell activation and thereby suppressing the immune response. Suppression of immune response has important clinical relevance, for example in transplantation surgery for preventing rejection episodes. Therefore, calcineurin as pharmacological target is of great importance and several attemps were made to develop agents which block the calcineurin signal pathway. Examples of such immunosuppressive drugs are FK506 (Fujisawa) and cyclosporine (Novartis) (Liu et al, 1991). These antibiotics inhibit calcineurin phosphatase activity in the presence of immunophilin receptor proteins (FKBP, cyclophillin) and thereby suppress immune response by preventing the activation of the T cell transcription factor NFAT (Liu et al., 1992), (Nelson et al., 1993). FK506 (tacrolimus) binds to the binding protein FKBP and thereby prevents calcineurin

from binding to FKBP. Accordingly the signal pathway is interrupted. No activation of the transcription factor NFAT is achieved and the T cell activation is disturbed.

Nevertheless, there are several severe disadvantages and side-effect of said drugs. In clinical trials with liver and renal transplant recipients it has been shown that FK506-based therapy was associated with increased toxicities in comparison to conventional therapy. Furthermore FK506 has negative effects on the bone mineral physiology.

Besides the role of the calcineurin signal pathway in immune response it has been shown that calcineurin is involved in apoptosis induction by glutamate excitotoxicity in neuronal cells (Ankarcrona et al., 1996). Low enzymatic levels of calcineurin have been associated with Alzheimers disease (Ladner et al., 1996), (Kayyali et al., 1997). Calcineurin inhibitors (FK506, Cyclosporin) prevented epileptogenesis in model organisms (Moriwaki et al., 1996). In the heart or in the brain calcineurin also plays a key role in the stress response after hypoxia or ischemia (Butcher et al., 1997), (Hashimoto et al., 1998), (Molkentin et al., 1998).

In summary, calcineurin is a crucial target to develop new substances suitable as drugs, especially as immunosuppressive drugs. Former screening systems using purified calcineurin and conventional assays like radioactive or HPLC assays (Klee, 1991), (Enz et al., 1994) did not lead to appropriate new substances. Therefore, the invention has the object to provide a new screening system for modulators of calcineurin taking advantage of new insights into the signal pathway of calcineurin. By the use of this new screening system it is possible to develop new pharmaceuticals with respect to the field of transplantation surgery, cardiac infarction and

apoplexy, chronic or acute neurodegeneration and inflammatory diseases, for example. This object is solved by a method according to claim 1. Preferred embodiments of the inventive method are depicted in the dependent claims 2 to 17. A kit, vectors, cells and a peptide suitable for performing the inventive method are claimed in claim 18 to 23. The wording of all claims is hereby made to the content of the specification by reference.

The inventive method is based on results showing that a physiological interaction between calcineurin and superoxide dismutase takes place which provides a suitable target for developing of a new screening system.

For a long time it was not understood why recombinant or even purified calcineurin exhibited only 1 to 2 % of the specific activity estimated in crude brain extracts until it was detected that the binuclear metal center of the enzyme contains a redoxsensitive Fe^{2+} (Yu et al., 1997). After calcium activation or during purification procedure the Fe^{2+} is oxidized by oxygen species and turns the enzyme inactive (Stemmer et al., 1995), (Wang et al., 1996).

Recently it has been shown that copper/zinc superoxide dismutase (CuZnSOD, EC 1.15.1.1) protects calcineurin against oxidative inactivation (Wang et al., 1996). The phosphatase activity of calcineurin is strongly dependent on the presence of calcium and calmodulin. The addition of Ca^{2+} in the presence of calmodulin leads to a drastic increase in activity. But during several minutes this activity is lost. By the addition of copper/zinc superoxide dismutase the activity can be maintained.

Superoxide dismutase (SOD) dismutates the hyperoxide anion (superoxide) into hydroperoxide and molecular oxygen. There are two forms of this enzyme: the mitochondrial form containing manganese and the cytosolic form containing copper and zinc. In general superoxide dismutase is considered to be a catcher of radicals and is discussed in the field of detoxification of reactive oxygen species. Therefore, the role of superoxide dismutase in the protection of the activity of calcineurin found by Wang et al. was considered to be the result of general redox function of superoxide dismutase. Now, surprising results of the inventor lead to the knowledge that a physiological interaction between calcineurin and superoxide dismutase takes place. Several mutants of copper/zinc superoxide dismutase lacking the enzymatic function showed the protective effect on the activity of calcineurin. That means that the effect of CuZnSOD is not due to the function of superoxide dismutase in redox regulation. These results teach that superoxide dismutase interacts physiologically with calcineurin and that CuZnSOD is one component of the calcineurin pathway which is important for the physiological functions of calcineurin.

These results are used to develop a new screening system for modulators of calcineurin in order to find inhibitors or activators of the calcineurin signal pathway. The inventive method is based on the complex formation between calcineurin and superoxide dismutase in the presence of potential modulators of this physiological interaction. If a potential modulator disturbs the complex formation, this substance is a good canditate for inhibiting the calcineurin signal pathway and could possibly be used as immunosuppressive drug, for example. On the other hand it could be favourable to identify a substance which promotes complex formation and thereby stimulates the calcineurin signal pathway, e.g. the T cell

response in result. Such a substance could be used in order to strengthen immune response. By the term "modulator" is meant any substance which influences the complex formation relating to the inventive method. Additionally is meant any substance which influences the interaction between calcineurin and its substrates, e.g. the peptide RII. Furthermore is meant any substance which influences the superoxide dismutase and/or calcineurin on the transcriptional, the translational and/or the posttranslational level.

Calcineurin as used in the inventive method is build up by the regulatory subunit A and the catalytic subunit B. The presence of both subunits is essential for physiological activity of calcineurin. Nevertheless, it is possible to perform the inventive method using only one of the subunits. There are several isoforms of calcineurin consisting of subunit calcineurin B and one out of the group comprising subunit calcineurin A-alpha, A-beta and A-gamma. Each isoform represents a special cell and tissue specific distribution. Therefore, the choice of isoform could be crucial for cell and tissue specifity of the substance to be screened. With respect to clinical application of the substances to be screened preferably human forms of the proteins are used.

Furthermore it is preferred to perform the inventive method in the presence of calmodulin and calcium, because the activity of calcineurin is dependent on these factors. Preferably the cytosolic form of superoxide dismutase containing copper and zinc is used for complex formation, because interaction between the mitochondrial form of superoxide dismutase containing e.g. manganese normally does not occur under physiological conditions. The complex formation is performed in the presence of at least one potential modulator of calcineurin or the calcineurin signal pathway, respec-

tively. The complex comprising calcineurin A, calcineurin B, superoxide dismutase and preferably calmodulin is the target for potential modulators which could stabilize or disturb the complex.

Advantageously, the complex formation is monitored during the whole process. It is possible to add the modulator before or after the complex formation has been performed. Preferably the modulator is added before complex formation because the effect of a weak modulator will possibly not be monitorable when complex formation has already finished.

In principle, there are two possibilities to monitor the complex formation. Firstly, the complex formation is directly monitored by the use of labeled components in the complex, preferably by fluorescence detection. Secondly, the complex formation is monitored by the activity of the complex, especially the enzymatic activity of calcineurin. This second method can be performed in addition to the firstly mentioned method or as an alternative. Clearly, the inventive method is not restricted by the method for detecting the influence of the modulator on complex formation.

In a preferred embodiment of the invention, a mixture of substances comprising at least one potential modulator is analyzed by the inventive method. By isolating the complex together with the possibly interacting modulator it is possible to separate the modulator out of the mixture and to identify it by common methods.

In one preferred embodiment of the invention the calcineurin and/or superoxide dismutase are labeled. Especially preferred is the use of fluorescent labels. Preferably, the labeled proteins are fusion proteins comprising a fluorescent prote-

in, e.g. enhanced green fluorescent protein (EGFP). These fusion proteins are provided by genetic engineering methods. It is also possible to label said proteins by other methods known to experts in the art, e.g. by the use of radioactive isotopes which are incorporated into the proteins.

Advantageously the components of the complex, i.e. calcineurin and superoxide dismutase are expressed in the cell, especially in an eukaryotic cell, as fluorescent fusion proteins. By the use of laser fluctuation correlation spectroscopy the complex formation of labeled proteins is monitored directly within the cell. This embodiment of the invention is described in greater detail in the example. The invention comprises several vectors useful for the expression of calcineurin and/or superoxide dismutase in eukaryotic cells. These vectors encode the proteins, especially CuZnSOD and the different subunits of calcineurin, as fusion proteins in connection with the fluorescent protein EGFP (enhanced green fluorescent protein). EGFP is only one example of possible labels useful in respect of the inventive method. Furthermore, the invention comprises cells, especially eukaryotic cells, stably transfected with the above-mentioned vectors thereby expressing superoxide dismutase and/or calcineurin. Preferably, these proteins are coexpressed, i.e. expressed within the same cell.

In an especially preferred embodiment, the genetic information of fusion proteins is integrated in the cell by homologous recombination. That means that the gene encoding the recombinant protein, especially the fluorescent fusion protein, is incorporated in the genome of the cell in the place of the naturally occuring gene. This leads to a cell essentially lacking the natural protein. By the use of such cells it is possible to identify modulators by the inventive method which

influence the transcriptional, translational or posttranslation level of calcineurin and/or superoxide dismutase expression.

In another embodiment of the inventive method the components of the complex are isolated and preferably purified before complex formation is performed in vitro. Advantageously, the proteins are provided with a tag in order to facilitate purification, e.g. a histidin (his) tag consisting of several histidines in sequence which permits affinity purification by known procedures. Corresponding vectors encoding the tagged proteins are comprised by the invention. These vectors are especially useful as prokaryotic expression vectors. Furthermore, the invention comprises cells bearing said vectors.

Advantageously, following purification of the his-tagged proteins the tag is excised by appropriate enzymatic digestion, e.g. by the use of cathepsin-C of carboxypeptidase-A. Especially preferred is the purification of calcineurin by ferro-nitrilotricacetat-metal (Fe-NTA) affinity chromatography and the purification of superoxide dimutase by copper/-zinc-nitrilotriacetat-metal (CuZn-NTA) affinity chromatography. Nevertheless, other purification procedures known to experts in the art are possible. Natural occuring protein could also be used in the inventive manner.

Besides the use in purification of calcineurin and/or superoxide dismutase Ni (nickel)-NTA, Fe-NTA and/or Cu/Zn-NTA is used to immobilize the his-tagged calcineurin and/or superoxide dismutase in order to isolate naturally occuring ligands of these proteins using this inventive matrix. By the term "ligand" is meant any low- or highmolecular endogenous, exogenous or synthetic substance which interacts with said proteins. This could be a peptide, protein, carbohydrate,

lipid, nucleic acid or a synthetic polymer, for example. These so-identified ligands are potential candidates for modulators of the calcineurin signal pathway.

When performing complex formation in vitro it could be preferred to add calmodulin and/or calcium to the reaction because these factors are necessary for enzymatic activity of calcineurin.

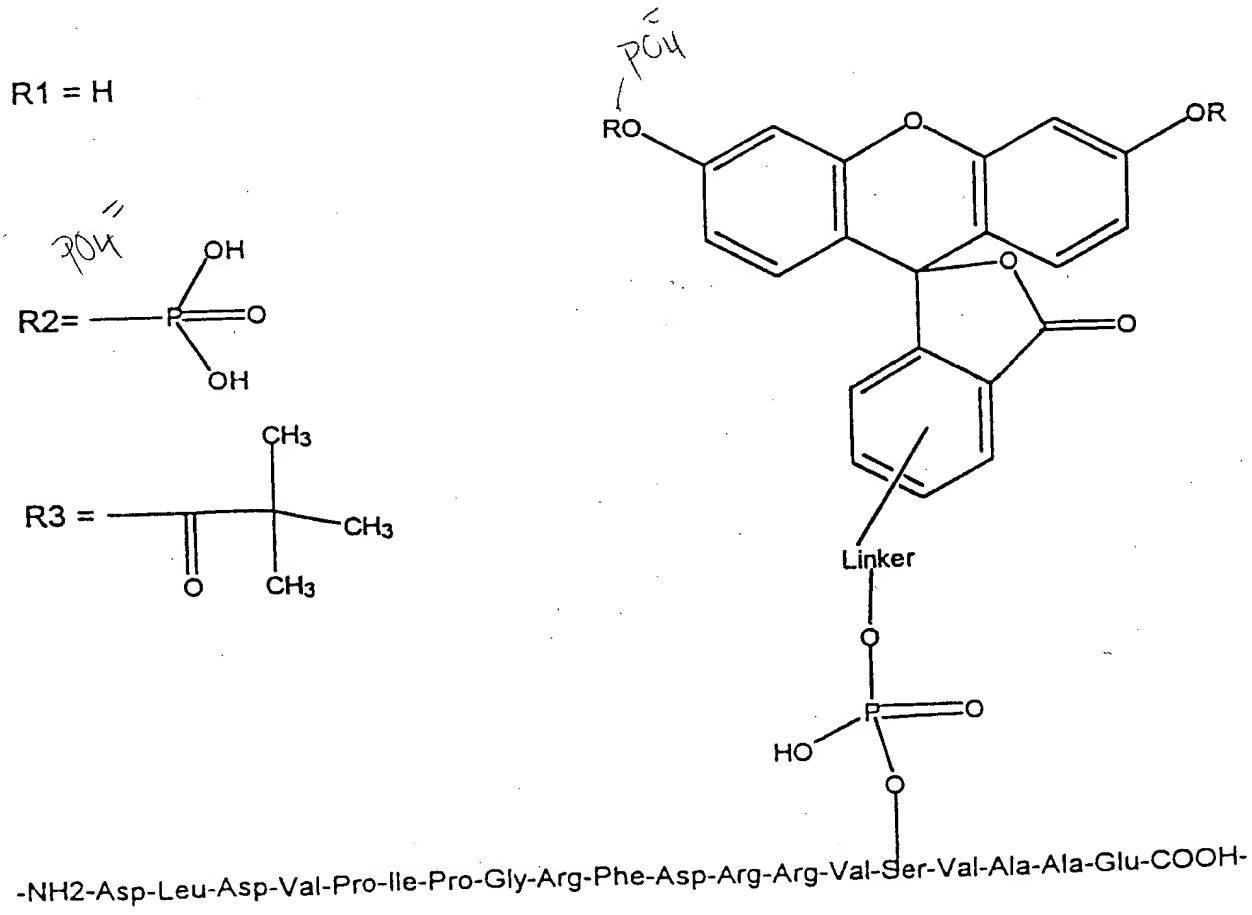
In another preferred embodiment of the invention the complex formation is monitored indirectly by analyzing the enzymatic activity of calcineurin. As outlined above the phoshatase activity of calcineurin is strictly dependent on the interaction with superoxide dismutase. Therefore, it is possible to monitor the complex formation indirectly by the measurement of phosphatase activity according to standard procedures. This is especially preferred if the laboratory equipment to perform fluorescent measurements as described above is not available. Furthermore, enzymatic analysis could be used in addition to fluorescence detection like laser fluctuation correlation spectroscopy, e.g. as control.

Preferably the enzymatic activity is analyzed by the use of a labeled substrate of calcineurin. The substrate is preferably labeled by fluorescence. One especially preferred substrate is the peptide RII characterized by the sequence:

Asp - Leu - Asp - Val - Pro - Ile - Pro - Gly - Arg - Phe - Asp - Arg - Arg - Val - Ser - Val - Ala - Ala - Glu.

In a preferred embodiment this peptide carries a fluorescent label at serine in position 15. This amino acid is

labeled with fluoresceine by incubating the peptide with fluoresceine-phosphoamidit, thereby providing a labeled substrate (RII-Fluophos). RII interacts with the active center of calcineurin, but it is not converted by the phosphatase. Hereby it is possible to label calcineurin in the active state. Furthermore, it is possible to phosphorylate RII-Fluophos at the fluoresceine moiety as depicted below at tyrosine residues. Due to the phosphorylation RII-Fluophos looses its fluorescence and thereby provides a phosphatase substrate which becomes fluorescent subsequent to dephosphorylation.



This peptide could be provided synthetically or it is expressed by cells, especially eucaryotic cells, which have been transfected with appropriate vectors encoding said peptide or other peptides useful as phosphatase substrates. In one embodiment of the invention the fluorescent peptide is used as peptide label in fluorescence microscopy. This provides another method in order to analyze the active state of the calcineurin/superoxide complex.

The inventive method as outlined above is suitable for developing a high-throughput bioassay to identify inhibitors and/or activators of the calcineurin signal pathway. Details are described in the example.

The invention comprises the use of inhibitors of activators of the calcineurin signal pathway for the treatment of acute and/or chronic neurological and cardiovascular diseases like Alzheimer, Parkinson, epilepsy, ischemia and heart-failure. Furthermore the use as immunosuppressive drugs, e.g. in the field of transplantation surgery and inflammatory diseases is included.

Finally the invention comprises a kit for screening of modulators of calcineurin. The kit provides calcineurin and superoxide dismutase enabling complex formation for the screening for modulators of calcineurin as described above. In a first embodiment of the kit the components of the complex are provided as proteins. This kit is suitable for performing the inventive method in vitro. In a second embodiment of the kit the proteins are provided in the form of vectors. These vectors have to be transformed/transfected into cells leading to the expressed proteins. These vectors are prokaryotic or eukaryotic expression vectors, respectively, and could be used to produce the proteins for the in

vitro assay or for the assay using complete cells as described above. In a third embodiment of the inventive kit cells transformed/transfected with the said vectors are provided saving the step of transforming/transfecting for the user. For details of the inventive kit reference is made to the above description.

The new approach to identify new substance classes of calcineurin/CuZnSOD inhibitors comprises inter alia:

- coexpression of CuZnSOD/calcineurin A and calcineurin B to generate a oxidative stable enzyme which is suitable for drug screening,
- efficient purification of CuZnSOD on CuZn-nitrilotriacetat-metal affinity chromatography to retain enzymatic activity,
- efficient purification of calcineurin on Fe-nitrilotriacetat-metal affinity chromatography to retain enzymatic activity and prohibit Fe^{2+} oxidation,
- identifying that mutations in the CuZnSOD associated with a neurological disorder (amyotrophic lateral sclerosis) are also critical for calcineurin-CuZnSOD interaction,
- use of fluorescent labeled recombinant CuZnSOD and calcineurin to screen for CuZnSOD/calcineurin activators or inhibitors,
- use of fluorescent labeled RII-peptide and calcineurin to screen for calcineurin activators or inhibitors,
- identification of calcineurin/CuZnSOD inhibitors or activators by using the recombinant enzymes as affinity ligands to purify new drugs from natural sources,
- inclusion of all isoforms, all known and two newly identified splicevariants into the screening procedure, which allows the identification of less toxic and tissue specific

drugs which are more suitable for the therapeutical treatment of different clinical indications.

The described features of the invention and further features result in greater detail from the examples in combination with the subclaims. The features could be realized in combination with each other or alone.

Example

1. Cloning of CuZnSOD transcript from human brain poly-A-RNA - Cloning of human CuZnSOD was performed by reverse transcription PCR using human brain poly-A-RNA as template (Clontech, Palo Alto, CA, USA). The oligonucleotides SODs1 5'-ttc cgt tgc agt cct cgg aac-3', SODas1 5'-taa ggg gcc tca gac tac atc-3', SOD-PQE60s2 5'-caa gcc atg gcg acg aag gcc gtg tgc gtg ctg-3', SOD-PQE60as2 5'-gaa gat ctt tgg gcg atc cca att aca cca c-3', SOD-PQE30-s2 5'- cgc gga tcc gcg acg aag gcc gtg tgc gtg -3' and SOD-PQE30-as2 5'-ggg ttc gaa tta ttg ggc gat ccc aat tac-3' were supplied by Interactiva (Ulm, Germany). Reverse transcription was performed with the SODas1 primer and 100 ng of poly-A-RNA according to the manufacturer's protocol (Expand reverse transcriptase, Boehringer Mannheim, Germany). The human CuZnSOD cDNA was amplified by nested PCR. The first PCR was performed in 20 µl, using 0,5 µl reverse transcription product, 10 µM SODs1 and SODas1 primers, 300 µM dNTPs, 2 µl of the manufacturer's 10 x PCR buffer and 2.5 U Taq-polymerase with 30 cycles of 1 min 95 ºC, 1 min 45 ºC, 1 min 72ºC followed by a second PCR (50 µl) with 5 µl of the purified first PCR product, 10 µM SOD-PQE60s2 and SOD-PQE60as2 primers, 300 µM dNTPs, 5 µl of the manufacturer's PCR buffer and 2,5 U Taq-polymerase with 30 cycles of 1 min 95 ºC, 1 min 60 ºC, 1 min 72ºC (Taq-polymerase, Pharmacia Biotech, Uppsala, Sweden). For the subcloning

into pQE30 expression vector the primers SOD-PQE30-s2 and SOD-PQE30-as2 were used instead of SOD-PQE60s2/SOD-PQE60as2.

2. Subcloning of human CuZnSOD into pQE60 expression vector (C-terminal fusion protein) - The SOD-pQE60 PCR product was purified by gel extraction prior to NcoI/BglII restriction (New England Biolabs). In order to generate a C-terminal histidin tag fusion protein the CuZnSOD transcript was ligated into the NcoI/BglII treated prokaryotic expression vector pQE60 (QIAexpress expression kit type IV and type ATG, Qiagen, Hilden, Germany). For selection, amplification and sequencing of the CuZnSOD vector construct (CuZnSOD-pQE60), 10 µl ligation product was transformed into E.coli M15[pREP4] cells (QIAexpress expression kit type ATG, Qiagen, Hilden, Germany). Correct reading frames and exclusion of mismatches were confirmed by radioactive and automated sequencing on both strands (T7-sequencing kit, Pharmacia Biotech, Uppsala, Sweden; ABI 377 sequencer, Applied Biosystems, USA).

3. Subcloning of human CuZnSOD into pQE30 expression vector (N-terminal fusion protein) - The SOD-pQE30 PCR product was purified by gel extraction prior to direct ligation into the pCR2.1 vector according to the manufacturer's protocols (TA-Cloning Kit, Invitrogen, De Schelp, Netherlands). After amplification and plasmid purification the pCR2.1-CuZnSOD vector construct was restricted with BamHI to yield a CuZnSOD transcript extended at the 3'-end with the sequence 5'-GAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCC-3' which originates from PCR2.1 vector and includes additional EcoRI/BstX-I/ SpeI/BamHI restriction sites. In order to generate a N-terminal histidin tag fusion protein the extended transcript was ligated into the BamHI/HindIII treated prokaryotic expression vector pQE30 (QIAexpress expression kit type IV, Qiagen, Hilden, Germany), blunted by incubation with Klenow-DNA-poly-

merase and circularized by a second treatment with T4-DNA-Ligase (Boehringer Mannheim, Germany). For selection, amplification and sequencing of the CuZnSOD vector construct (CuZn-SOD-pQE30), 10 µl ligation product was transformed into E.coli M15[pREP4] cells (QIAexpress expression kit type IV and type ATG, Qiagen, Hilden, Germany). Correct reading frames and exclusion of mismatches were confirmed by radioactive and automated sequencing on both strands (T7-sequencing kit, Pharmacia Biotech, Uppsala, Sweden; ABI 377 sequencer, Applied Biosystems, USA).

4. Site directed mutagenesis (point mutations associated with the neurological disorder Amyotrophic Lateral Sclerosis and important for calcineurin/CuZnSOD protein interaction) - Amino acid substitutions were introduced according to the manufacturer's protocol, using the primers SOD-PQE60-A4V (5'-caa gcc atg gcg acg aag gtc gtg-3'), SOD-A4V (5'-tcc gcg acg aag gtc gtg tgc gtg ctg-3'), SOD-G37R (5'-gg aag catt aaa aga ctg act gaa ggc-3'), SOD-D90A (5'aat gtg act gct gcc aaa gat ggt gtg-3'), SOD-G93A (5'-gct gac aaa gat gct gtg gcc gat gtg-3'), SOD-AflIII (5'-acg cag gaa aga aca tgt gag caa aag-3'), SOD-BglII (5'-acg cag gaa aga aga tct gag caa aag-3') and the expression vector CuZnSOD constructs CuZn-SOD-pQE30 and CuZnSOD-pQE60, respectively (Chameleon site directed mutagenesis kit, Stratagene, San Diego, CA, USA). Incorporation of the site-directed mutations was confirmed by DNA sequencing of the expression vector. Site directed mutagenesis yielded eight additional vector sequences corresponding to eight protein sequences with clinical relevant amino acid substitutions:

Vector-construct	**amino acid subst. (pos. in protein)**	**nucleic acid subst. (pos. in sequ. prot.)**
CuZnSOD-pQE60	WT = wild-type	= SEQ ID NO 15

CuZnSOD-pQE60-(A4V)	Ala-4 -> Val-4	c-128 -> t-128	
CuZnSOD-pQE60-(G37R)	Gly-37 -> Arg-37	g-226 -> a-226	
CuZnSOD-pQE60-(D90A)	Asp-90 -> Ala-90	a-386 -> c-386	
CuZnSOD-pQE60-(G93A)	Gly-93 -> Ala-93	g-395 -> c-395	

CuZnSOD-pQE30	WT = wild-type = SEQ ID NO 13		
CuZnSOD-pQE30-(A4V)	Ala-4 -> Val-4	c-161 -> t-161	
CuZnSOD-pQE30-(G37R)	Gly-37 -> Arg-37	g-259 -> a-259	
CuZnSOD-pQE30-(D90A)	Asp-90 -> Ala-90	a-419 -> c-419	
CuZnSOD-pQE30-(G93A)	Gly-93 -> Ala-93	g-428 -> c-428	

5. Recombinant expression and purification of wild-type and mutated CuZnSOD - The CuZnSOD-pQE60 or CuZnSOD-pQE30 vector transformed E.coli M15[pREP4] cells were plated on LB / ampicillin (100 µg/ml) / kanamycin (25 µg/ml) agar. Expression cultures were grown in 250 ml LB / ampicillin (100 µg/ml) / kanamycin (25 µg/ml) until the OD_{600} was 0.6. Constitutive leakage expression of human CuZnSOD was fully prevented by the repressor plasmid pREP4-lacI. Production of the human CuZnSOD fusion proteins was induced by addition of IPTG (1 mM). After two hours the bacterial cells were harvested by centrifugation (4000 g, 20 min), resuspended in 8 ml buffer A (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 500 mM NaCl) and homogenized by three freeze thaw cycles and sonication on ice (Bandelin sonoplus GM70, 300 W, 3 x 10 sec). The lysate was centrifuged (10.000 g, 20 min) and incubated with 750 µl CuZn-NTA (nitrilotriacetat)-agarose for batch affinity binding for 1 h at 4 ºC (Qiagen expressionist kit, Qiagen, Hilden, Germany). CuZn-NTA-agarose was prepared from Ni-NTA-agarose (Qiagen expressionist kit, Qiagen, Hilden, Germany) by subsequent washes in:

1) 2 volumes of bidestilled water
2) 3 volumes of regeneration buffer (6 M guanidiniumhydrochloride, 0.2 M acetic acid)

3) 5 volumes bidestilled water
4) 3 volumes 2% SDS
5) 1 volume 25% ethanol
6) 1 volume 50% ethanol
7) 1 volume 75% ethanol
8) 5 volumes 100% ethanol
9) 1 volume 75% ethanol
10) 1 volume 50% ethanol
11) 1 volume 25% ethanol
12) 1 volume bidestilled water
13) 5 volumes 100 mM Na-EDTA pH 8.0
14) 5 volumes bidestilled water
15) 2 volumes 100 mM $CuSO_4$ / 100 mM $ZnSO_4$ / 1 mM reduced glutathion / 1 mM dithiothreitol
16) 2 volumes bidestilled water
17) 2 volumes regeneration buffer (6 M guanidiniumhydrochloride, 0.2 M acetic acid)
18) 2 volumes buffer buffer A2 (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 500 mM NaCl, 200 µM $CuSO_4$ / 200 µM $ZnSO_4$ / 1 mM reduced glutathion / 1 mM dithiothreitol

The batch was applied to a 30 ml chromatography column, washed with 15 ml buffer A (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 500 mM NaCl) and subsequently with 8 ml buffer B (20 mM Tris-HCl pH 7.9, 60 mM imidazole, 500 mM NaCl). C-terminal or N-terminal histidin tagged CuZnSOD was eluted three times with 1,2 ml buffer C (10 mM Tris-HCl, 500 mM imidazole, 250 mM NaCl). Purity and correct expression products were checked by immunoblotting or N-terminal protein sequencing after separation of 20 µl eluate in SDS-PAGE (discontinuous 12,5 % SDS-PAGE). To examine the protein levels in bacterial culture all CuZnSOD variants were induced synchronously at OD_{600} = 0.6 with 1 mM IPTG. After 1 h, 2 h, 3 h, 4 h and 20 h, aliquots (1 ml) of E.coli cultures were

taken, centrifuged and homogenized in buffer A as described. The pellet was resuspended in 1 ml H_20. Subsequently, 20 µl of the supernatant (soluble fractions) or 20 µl of the sonicated pellet suspension (insoluble fractions) were mixed with 7 µl of denaturing sample buffer (10 % SDS, 10 % mercaptoethanol, 20 % glycerol, 130 mM Tris-HCl pH 6.8, 0.03 % bromphenol blue). The samples were heated for 2 minutes at 80 °C and analyzed by 12 % SDS-PAGE. After coomassie staining, the electropherograms were digitized with a CCD camera (Gel Doc 1000, BioRAD) and analyzed by densitometry using NIH-Image software (1.61).

6. Processing of CuZnSOD - In order to remove the nonphysiological histidin tag and to yield CuZnSOD useful for clinical applications the N-terminal histidin tagged CuZnSOD was proteolytically processed with cathepsin-C or the C-terminal variant was processed with carboxypeptidase-A according to the manufacturer's protocols (Boehringer-Mannheim, Mannheim, Germany). Treatment with cathepsin-C yielded a processed CuZnSOD starting with the amino acids NH_2-GSAT KAVCVLKGDGP (indicated in sequence protocol CuZnSOD-pQE30 SEQ ID NO 13). C-terminal fusion protein was yielded the C-terminal amino acid sequence VIGIAQR-COOH (indicated in sequence protocol CuZnSOD-pQE30 SEQ ID NO 13). Verification was done by peptide sequencing.

7. Reactivation of CuZnSOD - In order to yield physiologically relevant active homodimeric CuZnSOD, the CuZn-NTA eluate was ultrafiltered through a 5 kD membrane (Omegacell, Filtron, Northborough, MA, USA). For buffer exchange the samples were washed three times in reconstitution buffer (50 mM sodium citrate pH 5.5, 1 mM DTT). The protein solutions were incubated at 8 ºC for 7 days (250 µg/ml protein). After distinct time intervals aliquots of the refolding mixture

were either analyzed by native gel electrophoresis (2.6 µg CuZnSOD) and activity staining or assayed in a spectrophotometer (0.5 - 1 µg CuZnSOD, superoxide dismutase assay kit, Calbiochem, San Diego, CA, USA). For visualization of protein bands native gels were stained with coomassie blue. For the production of larger CuZnSOD amounts M15-E.coli cells were subsequently grown in 15 ml, 200 ml, 2500 ml and 20 L flasks. Refolded CuZnSOD proteins were dialyzed against 100 volumes of buffer D (10 mM Tris-HCl 0.1 % Saccharose) and lyophylized.

8. SOD assay and activity staining - Enzymatic activity of the CuZnSOD proteins were either analyzed by 10 % native gel electrophoresis and activity staining with nitrotetrazolium blue dye or by a quantitative spectrophotometrically assay according to published protocols (Beauchamp and Fridovich, 1971; Nebot et al., 1993). Protein yields were determined by the Bradford method (Protein assay kit, BioRAD, Hercules, CA, USA). The concentration of purified CuZnSOD was determined spectrophotometrically using the extinction coefficient $\varepsilon_{265} = 1.84 \times 10^4\ M^{-1}\ cm^{-1}$.

9. Subcloning of human CuZnSOD into pEGFP eukaryotic expression vector and generation of stable transfected PC12 cells (C-terminal fusion protein with enhanced green fluorescent protein as a fluorescent marker/label) - Using 10 µM of the primers SOD-pEGFP-s 5'-ccg cgg gcc cgc cat ggc gac gaa ggc cgt gtg cgt gc-3' and SOD-pEGFP-as 5'-gct cac cat ggt ggt ttg ggc gat ccc aat tac acc ac-3', 10 ng CuZnSOD-pQE60 vector, 300 µM dNTPs, 5 µl of the manufacturer's PCR buffer and 2,5 U Taq-polymerase with 25 cycles of 1 min 95 ºC, 1 min 60 ºC, 1 min 72ºC (50 µl total volume, Taq-polymerase, Pharmacia Biotech, Uppsala, Sweden) a PCR product was generated which was cleaved by ApaI/NcoI digestion. The purified

PCR product was ligated into ApaI/NcoI treated pEGFP-N3 vector (Clontech Laboratories, Palo Alto, CA, USA). After amplification in XL2-Blue cells (25 µg/ ml kanamycin) and plasmid purification the CuZnSOD-pEGFP vector construct was transfected into PC12 rat adrenal pheochromocytoma cells using the CalPhos™ Transfection Kit according to the manufacturer's protocols (Clontech Laboratories, Palo Alto, CA, USA). Stable transfected CuZnSOD-pEGFP clones were selected by fluorescence microscopy (exitation 488 nm/ emission 520 nm, MRC 1024 confocal microscope, BioRAD Laboratories, Hercules, CA, USA).

10. Cloning of the regulatory subunit human calcineurin-B - Cloning of human calcineurin-B was performed by reverse transcription PCR using human brain poly-A-RNA as template (Clontech, Palo Alto, CA, USA). The oligonucleotides CNBa-s1 5'-ccg ccg acc cgc cga gca-3', CNBa-as1 5'-ggt act ctc tga taa gag-3', CNBa-s3 5'-gga att ccc cgg gga aag agg aga aat taa cta tgg gaa atg agg caa gtt atc-3', CNBa-as2 5'-ttc cgg gcc caa gct tct aat taa tca cac atc tac cac cat c-3' were supplied by Interactiva (Ulm, Germany). Reverse transcription was performed with the CNBa-as1 primer and 100 ng of poly-A-RNA according to the manufacturer's protocol (Expand reverse transcriptase, Boehringer Mannheim, Germany). The human calcineurin-B cDNA was amplified by nested PCR. The first PCR was performed in 20 µl, using 0.5 µl reverse transcription product, 10 µM CNBa-s and CNBa-as1 primers, 300 µM dNTPs, 2 µl of the manufacturer's 10 x PCR buffer and 2.5 U Pfu-polymerase with 20 cycles of 1 min 95 ºC, 1 min 55 ºC, 2 min 72 ºC followed by a second PCR (50 µl) with 5 µl of the purified first PCR product, 10 µM CNBa-s3 and CNBa-as2 primers, 300 µM dNTPs, 5 µl of the manufacturer's PCR buffer and 2.5 U Pfu-polymerase with 20 cycles of 1 min 95 ºC, 1 min

55 ºC, 1 min 72ºC (Pfu-polymerase, Stratagene, San Diego, CA, USA).

11. Cloning of the catalytic subunit human calcineurin-A-Alpha and splicevariants - Cloning of human calcineurin-A-alpha was performed by reverse transcription PCR using human brain poly-A-RNA as template (Clontech, Palo Alto, CA, USA). The oligonucleotides CNAa-s1 5'-gcg tcg ctg tcc tcc ggc agc-3', CNAa-as1 5'-gtg aac agg aag tgg tca ctg-3', CNAa-s2 5'-cat gcc atg gatc cat gtc cga gcc caa ggc-3', CNAa-as4 5'-tcc ccc cgg ggta ccc tag tta atc act gaa tat tgc tgc tat tac-3' were supplied by Interactiva (Ulm, Germany). Reverse transcription was performed with the CNAa-as1 primer and 100 ng of poly-A-RNA according to the manufacturer's protocol (Expand reverse transcriptase, Boehringer Mannheim, Germany). The human calcineurin-A-Alpha cDNA was amplified by nested PCR. The first PCR was performed in 25 µl, using 0,5 µl reverse transcription product, 10 µM CNAa-s1 and CNAa-as1 primers, 200 µM dNTPs, 2.5 µl of the manufacturer's 10 x PCR buffer and 1.25 U Pfu-polymerase with 30 cycles of 40 seconds at 95 ºC, 40 seconds at 55 ºC, 3 min 72 ºC followed by a second PCR (25 µl) with 2.5 µl of the purified first PCR product, 10 µM CNAa-s2 and CNAa-as2 primers, 200 µM dNTPs, 2.5 µl of the manufacturer's PCR buffer and 2.5 U Pfu-polymerase with 25 cycles of 40 seconds at 95 ºC, 40 seconds at 55 ºC, 3 min 72 ºC (Pfu-polymerase, Stratagene, San Diego, CA, USA).

Hereby a new splicevariant was identified, which is important for calcium regulation and proteolytic regulation of calcineurin-A. The splicevariant lacks the hole catalytic phosphatase domain and part of calcineurin-binding-site (Elimination of nucleic bases 208-1317 in sequence protocol

CNAa1-pQE30 SEQ ID NO 17). The corresponding vector is named CNAa3-pQE30:

Location/Qualifiers
151..606 /note="splicevariant: Calcineurin A alpha 1 lacking phosphatase domain, newly generated N-terminus exhibits protease activity"
115..150 /note="His-Tag"
649..1161 /note="Calcineurin B;Calcineurin B alpha Ca^{2+} binding"

12. Cloning of the catalytic subunit human calcineurin-A-Beta and splicevariants - PCR was performed as described under 11. with the exception that the primers CNAb-s1 5'-gag cct agc cga gcc ccg gg-3' and CNAb-as1 5'-ctg gga agt agt ggg tca ctg-3' were used for the first PCR and the primers and CNAb-s2 5'- cat gcc atg gat cca tgg ccg ccc cgg agc c-3' and CNAb-as4 5'- tcc ccc cgg ggt acc cta gtt aat cac tgg gca gta tgg ttg cca g-3' were used for second PCR.

13. Cloning of the catalytic subunit human calcineurin-A-Gamma and splicevariants - PCR was performed as described under 11. with the exception that the primers CNAg-s1 5'-gga gcc tgg agg agg ccg ag-3' and CNAg-as1 5'-cgg cag gac tct aag tca tga-3' were used for the first PCR and the primers and CNAg-s2 5'-cat gcc atg gat cca tgt ccg gga ggc gct tc-3' and CNAg-as4 5'-tcc ccc cgg ggt acc cta gtt aat cat gaa tgg gct ttc ttc cct t-3' were used for second PCR.

Hereby a new splicevariant was identified, which is important for calcium regulation and proteolytic regulation of calcineurin-A. The splicevariant with human exon is not yet available in gene database (substitution of nucleic bases 1474-1503 in sequence protocol CNAg2-pQE30 SEQ ID NO 32) with

5'-ACA GTA GAA GCG GTA GAG GCC CGG GAA GCC-3' (corresponding peptide: NH_2-TVEAVEAREA-COOH). The corresponding vector is named CNAg3-pQE30.

Location/Qualifiers
115..150 /note="His-Tag"
151..1689 /note="Calcineurin-A-Gamma-2"
1474..1503 /note="human brain calcineurin-A-gamma alternative exon = interaction domain with cytoskelett, death-domain homolog, stomatin homolog"
1690..1731 /note="RBS&MCS2"
1732..2244 /note="Calcineurin-B"

14. Subcloning of calcineurin-B and calcineurin-A variants into pQE30 - For the recombinant expression in procaryotic cells calcineurin-B was subcloned with either calcineurin-A-alpha1, calcineurin-A-alpha2, calcineurin-A-beta1, calcineurin-A-beta2, calcineurin-A-gamma1 or calcineurin-A-gamma2. The purified calcineurin-A-alpha, calcineurin-B-alpha or calcineurin-A-gamma PCR products (described in 11. - 13.) were restricted with BamHI/XmaI. The purified calcineurin-B product (described in 10.) was restricted with XmaI/HindIII and ligated together with the respective calcineurin-A-fragment into the BamHI/HindIII treated vector pQE30 to yield the final procaryotic expression vector constructs CNAa1-pQE30, CNAa2-pQE30, CNAa3-pQE30, CNAb1-pQE30, CNAb2-pQE30, CNAg1-pQE30, CNAg2-pQE30 and CNAg3-pQE30.

15. Recombinant coexpression and purification of calcineurin-B/calcineurin-A heterodimers with CuZnSOD - CNAa1-pQE30, CNAa2-pQE30, CNAa3-pQE30, CNAb1-pQE30, CNAb2-pQE30, CNAg1-pQE30, CNAg2-pQE30 or CNAg3-pQE30 were transformed into E.coli M15[pREP4][CuZnSOD-pQE30] to yield cells able to coexpress calcineurin-A, calcineurin-B and CuZnSOD. cells

were plated on LB / ampicillin (100 µg/ml) / kanamycin (25 µg/ml) agar. Expression cultures were grown in 250 ml LB / ampicillin (100 µg/ml) / kanamycin (25 µg/ml) until the OD_{600} was 0.6. Constitutive leakage expression was prevented by the repressor plasmid pREP4-lacI. Production of the human calcineurin-A/calcineurin-B histidin tagged heterodimers was induced by addition of IPTG (1 mM). After four hours the bacterial cells were harvested by centrifugation (4000 g, 20 min), resuspended in 8 ml buffer A (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 500 mM NaCl) and homogenized by three freeze thaw cycles and sonication on ice (Bandelin sonoplus GM70, 300 W, 3 x 10 sec). The lysate was centrifuged (10,000 g, 20 min) and incubated with 750 µl Fe-NTA-agarose for batch affinity binding for 1 h at 4 ºC (Qiagen expressionist kit, Qiagen, Hilden, Germany). Fe-NTA-agarose was prepared from Ni-NTA-agarose (Qiagen expressionist kit, Qiagen, Hilden, Germany) by subsequent washes in:

1) 2 volumes of bidestilled water
2) 3 volumes of regeneration buffer (6 M guanidiniumhydrochloride, 0.2 M acetic acid)
3) 5 volumes bidestilled water
4) 3 volumes 2% SDS
5) 1 volume 25% ethanol
6) 1 volume 50% ethanol
7) 1 volume 75% ethanol
8) 5 volumes 100% ethanol
9) 1 volume 75% ethanol
10) 1 volume 50% ethanol
11) 1 volume 25% ethanol
12) 1 volume bidestilled water
13) 5 volumes 100 mM Na-EDTA pH 8.0
14) 5 volumes bidestilled water
15) 2 volumes 100 mM $FeSO_4$ / 1 mM reduced glutathion / 1 mM dithiothreitol/ 100 mM ascorbic acid

16) 2 volumes bidestilled water

17) 2 volumes regeneration buffer (6 M guanidiniumhydrochloride, 0.2 M acetic acid)

18) 2 volumes buffer buffer A3 (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 500 mM NaCl, 200 μM $FeSO_4$ / 1 mM reduced glutathion / 1 mM dithiothreitol/ 1 mM ascorbic acid

The batch was applied to a 30 ml chromatography column, washed with 15 ml buffer A4 (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 500 mM NaCl/ 1 mM reduced glutathion / 1 mM dithiothreitol/ 1 mM ascorbic acid) and subsequently with 8 ml buffer B (20 mM Tris-HCl pH 7.9, 60 mM imidazole, 500 mM NaCl/ 1 mM reduced glutathion / 1 mM dithiothreitol/ 1 mM ascorbic acid). N-terminal histidin tagged calcineurin-A/calcineurin-B heterodimer was eluted three times with 1.2 ml buffer C (10 mM Tris-HCl, 500 mM imidazole, 250 mM NaCl/1 mM reduced glutathion / 1 mM dithiothreitol/ 1 mM ascorbic acid, buffer was degased and subsequently saturated with nitrogen). To prevent oxidation of calcineurin, the eluate was stored at -80 ºC in nitrogen containing and oxygen free vials. Purity and correct expression products were checked by immunoblotting or N-terminal protein sequencing after separation of 20 μl eluate in SDS-PAGE (discontinuous 12,5 % SDS-PAGE).

16. Subcloning of human calcineurin-A-Alpha into pEGFP eukaryotic expression vector and generation of stable transfected PC12 cells (C-terminal fusion protein with enhanced green fluorescent protein as a fluorescent marker) - The vector CNAa2-pQE30 was digested with BamHI/XmaI to generate a sticky end CNAa2 fragment. The purified fragment was ligated into Bgl-II/XmaI treated pEGFP-C1 vector (Clontech Laboratories, Palo Alto, CA, USA). After amplification in XL2-Blue cells (25 μg/ ml kanamycin) and plasmid purification the

CNAa-pEGFP vector construct was transfected into PC12 rat adrenal pheochromocytoma cells using the CalPhos™ Transfection Kit according to the manufacturer's protocols (Clontech Laboratories, Palo Alto, CA, USA). Stable transfected CNAa-pEGFP clones were selected by fluorescence microscopy during a three month propagation procedure (exitation 488 nm/emission 520 nm, MRC 1024 confocal microscope, BioRAD Laboratories, Hercules, CA, USA).

17. Subcloning of calcineurin-A-Beta into pEGFP - The same procedure as described in 16. was applied except that the CNAa2-pQE30 vector was substituted by CNAb2-pQE30 to generate CNAb-pEGFP.

18. Subcloning of calcineurin-A-Gamma into pEGFP - The same procedure as described in 16. was applied except that the CNAa2-pQE30 vector was substituted by CNAg2-pQE30 to generate CNAg-pEGFP.

19. Western blotting and protein sequencing - Transfer of purified proteins from 12% SDS-PAGE to PVDF membranes (Boehringer-Mannheim, Mannheim, Germany) was performed according to standard protocols using transfer buffer (48 mM Tris, 39mM Glycine, 20% methanol, 1% SDS, pH 9.2) and following blotting conditions: 75 min at 25 V/110 mA. Blocking, washing and detection (HRP detection system) were performed according to the manufacturer's protocols (ECL kit, Amersham, Buckinghamshire, UK). An anti-human CuZnSOD antibody (1:5,000 dilution, rabbit polyclonal anti-human SOD1 antibody; BIOMOL, Hamburg, Germany) was used as primary antibody and an anti-rabbit IgG antibody (1:10,000 dilution) labeled with HRP was used as secondary antibody. For the detection of calcineurin-A (alpha, beta, gamma isoforms) a polyclonal calcineurin-A antibody was used as 1:5000 dilution (Sigma Aldrich, Deisen-

hofen, Germany). For N-terminal protein sequencing the PVDF membrane was soaked in 100% methanol. Proteins which seemed to be blocked by N-terminal posttranslational modifications were treated with acylamino-acid-peptidase according to the manufacturer's protocol (Boehringer-Mannheim, Mannheim, Germany). Coomassie brilliant blue stained bands were cut out. Automated Edman degradation of peptides was performed on an Applied Biosystems protein sequencer (476A).

20. Calcineurin phosphatase assay - 100 ng - 4 µg recombinant calcineurin (calcineurin-A/B heterodimer), 100 ng - 1 µg purified bovine brain calcineurin (Sigma Aldrich, Deisenhofen, Germany) or 100 µg homogenized tissue or cell extracts were used for classical calcineurin phosphatase assays. 100 µg cells or tissue were homogenized exactly as described (Stemmer et al., 1995). Partly purified and redox sensitive calcineurin was prepared by centrifugation at 14,000 rpm at 4°C for 10 min (Eppendorf Centrifuge 5417R) and the resulting supernatant was separated on a 1.5 x 10 cm Sephadex-G50 gelfiltration column as described (Stemmer et al., 1995), (Gold et al., 1997). Phosphotyrosine phosphatase assay was performed in microplates (100 µl total assay volume) either using 30 µM fluoresceinmonophosphate or 20 mM para-nitrophenylphosphate (Sigma Aldrich, Deisenhofen, Germany); 10 µl recombinant, purified or partly purified and assay buffer (25 mM Tris/HCl, pH 7; 2 mM $CaCl_2$; 0.1 µM calmodulin; 25 µM FK506). After starting the enzymatic reaction with para-nitrophenylphosphate or fluoresceinmonophosphate the absorbance at 405 nm (para-nitrophenylphosphate) or fluorescence (λ exication = 485 nm; λ emission = 520 nm) was monitored over 20 min at 30°C using a UV/VIS/fluorescence microplate photometer (Biolumin 960 kinetic fluorescence/absorbance photometer, Molecular Dynamics). Phosphoserine phosphatase assay was performed as described (Hubbard and Klee, 1991), (Wang et

al., 1996). In short: 40 µl recombinant or partly purified calcineurin was mixed with test buffer (40 mM Tris/HCl pH 8; 0.1 M KCl; 0.4 mg/ml BSA; 0.67 mM DTT; 0.67 µM calmodulin; 1 µM FKBP binding protein; 0.5 µM ocadaic acid for inhibition of phophatase A1 and A2) and enzymatic reaction and calcium induced redox-inactivation of calcineurin started by addition of 20 µl substrate buffer (7.7 µM radioactive phosphorylated RII-peptid, 2.0 mM $CaCl_2$). The assay was performed in duplicates and the addition of 1 µM FK506 or cyclosporine was used to verify calcineurin activity for each reading point. The protective effect of CuZnSOD against redox inactivation of calcineurin was determined by addition of 3 µg recombinant human wild-type or mutated CuZnSOD (constant CuZnSOD protein) or addition of 1.67 units of recombinant human wild-type or mutated CuZnSOD (constant CuZnSOD activity). The reaction mixture was incubated for 2 min at 30°C and stopped with 100 mM potassium phosphate / 5% TCA. The reaction mixture was passed through a 0.5 ml ion-exchange column (Dowex; AG 50W-X8, BioRad) and the unbound phosphate eluted with 0.5 ml water. The quantity of released phosphate was determined by a scintillation counting.

An enzymatic protein phosphatase assay was established using the nonphysiological substrate fluoresceinmonophosphate (FMP). Assuming a Michaelis-Menten kinetic for FMP and using the Lineweaver-Burk method for analysis of kinetic data a K_M of 40 µM and a V_{max} of 400 µmol / min was determined. The assay was applicable to calcineurin and magnesium dependent proteinphosphatase 2C (data not shown, (Grothe et al., 1998)). The enzymatic activity is linear in the range of 12.5 pM to 75 pM calcineurin. FMP is more sensitive than para-nitrophenylphosphate (pNPP). Neither FMP nor pNPP are useful to measure calcineurin activity in crude preparations by inhibition with the immunsuppressive drugs FK506 or cyclosporine

(cell homogenate, partly purified calcineurin). Both substrates also failed to measure calcium induced redox-inactivation of calcineurin or CuZnSOD mediated protection of this inactivation. The inhibition assay also failed when calcium was substituted against other divalent cations (Ni^{2+}, Mg^{2+}). Only the physiological relevant substrate could be used in an immunsuppressive drug inhibitory assay (RII-peptide phosphopeptide). In the classic radioactive assay 95% inhibition with 1 μM FK506 or cyclosporine was determined. It is concluded that inhibition of calcineurin activity by immunosuppresive drugs needs larger molecular weight substrates than pNPP and FMP. Furthermore it is concluded that redoxsensitivity is linked to phosphoserine phosphatase activity and therefore not detectable with phosphotyrosine analoges like pNPP or FMP. The recombinant human wild-type CuZnSOD and purified human erythrocyte CuZnSOD (Sigma Aldrich, Deisenhofen, Germany) were effective to protect 50-100% of calcineurin after calcium induced redox inactivation. Mutated CuZnSOD proteins, associated with the severe neurological disorder amyotrophic lateral sclerosis, were less effective to protect calcineurin against redox inactivation.

Protective effect of CuZnSOD of calcium induced inactivation of calcineurin

Not Fluor

Percentage of FK506 inhibitable RII-phosphopeptide activity after 20 min compared with the activity at 0 min

human CuZnSOD	**constant protein (3 μg)**	**constant activity (1.67 U)**
erythrocyte wild-type (8330 U/mg)	57 +/-10 %	57 +/-10 %
recombinant wild-type	70 +/-33 %	58 +/-22 %

(6380 U / mg)		
recomb. mutation D90A (4590 U/ mg)	42 +/-17 %	32 +/-15 %
recomb. mutation G93A (2130 U/ mg)	16 +/-16 %	21 +/-22 %
recomb. mutation A4V (1820 U/ mg)	22 +/-27 %	8 +/-3 %
control (no CuZnSOD) (0 U / mg)	9 +/-7 %	9 +/-7 %

The protective effect does not depend on CuZnSOD activity since higher protein amounts of mutated CuZnSOD corresponding to a higher enzymatic activity were even less effective in protection of calcineurin.

Therefore it is concluded that amino acid substitutions, associated with familial amyotrophic lateral sclerosis, are important for the protein interaction of calcineurin and CuZnSOD and therefore are involved in the CuZnSOD mediated protection of calcium induced redox inactivation of calcineurin. Since this protective effect is disturbed in amyotrophic lateral sclerosis and protection of calcineurin by CuZnSOD it may also be important in other neurological and cardiovascular diseases (Alzheimer, Parkinson, epilepsy, ischemia, heart-failure).

An high-throughput bioassay was developed to detect and isolate artificial or endogenous drugs enhancing (activators) CuZnSOD-calcineurin interaction and therefore protecting calcineurin against redox-inactivation or drugs reducing (inhibitors) CuZnSOD-calcineurin interaction and therefore inhibit calcineurin activity. Inhibitors are useful to substitute toxic immunsuppressive drugs like FK506 or cyclosporine. Activators and inhibitors may be useful for the

therapeutical treatment of amyotrophic lateral sclerosis, Parkinson, Alzheimer, epilepsy, ischemia and cardiovascular diseases.

21. High Throughput BioAssay using recombinant calcineurin-A, recombinant calcineurin-B, calmodulin and recombinant CuZnSOD (analytical assay to identify activators or inhibitors of CuZnSOD/calcineurin interaction) - Laser fluctuation correlation spectroscopy (FCS) is a useful tool to quantify ligand-ligand interactions. The fluorescence F(t) of a optical well defined volume element which is excited by a confocal laser is monitored as a function of time. The temporal autocorrelation of the fluorescence fluctuation δ F(t) yields the time scale of this dynamics and the average number of independent fluorophores in the probe volume. If the fluorescene fluctuation arise from diffusive motion and from fluorescence sensitive reaction, fluorescent fluctuation correlation function signal is approximated by the formula:

$$G_{DR}(\tau) = G_{Diff}(\tau) * [1+A * \exp(-k_R * \tau)]$$

τ = fluorescence correlation time

k_R = apparent binding constant of the fluorescent labeled ligand

A = equilibrium coefficent dependent constant

If one measurement is performed with a solution only containing the fluorescent labeled ligand and a second measurement is performed with a solution containing the fluorescent labeled ligand and an interacting molecule the correlation function G_{DR} can be separately analyzed and yields binding parameters of the interacting molecules. Upon binding of the ligand to the interacting molecule the hydrodynamic radius increases and therefore the diffusion coefficient decreases resulting in a longer correlation time.

A fluorescence labeled recombinant CuZnSOD as a fluorescent label was used to monitor the binding dynamics to calcineurin. CuZnSOD was labeled with Oregon-Green-514 dye according to the manufacturer's protocols (FluoReporter Protein labeling Kit, Molecular Probes, Leiden, Netherlands). The amount of fluorescent dye labels per CuZnSOD dimer was quantified by determining the ratio of the absorbance at 265 nm (CuZnSOD protein) / 514 nm (Oregon-dye). The diffusion constant and correlation time of the labeled CuZnSOD (100 nM) was measured on a bovine serum albumin treated glass plate with an confocal laser microscope attached to an autocorrelator (λ excitation = 488 nm, λ emission = 511 nm) in 10 μl assay buffer containing 50 mM sodiumphosphate pH 7.1, 150 mM NaCl, 0.67 mM DTT, 0.67 μM calmodulin, 0.67 mM $CaCl_2$, 1 mM $MgCl_2$. The beam from a modelocked Ti:Sa or cw argon ionlaser was collimated to fill the back aperture of a immersion microscope objective (Zeiss C-Apochromat 63x1.2w), producing a small diffractionlimited spot. The emitting fluorescent light was collected by the same objective separated from the excitation light by a beamsplitter/filter combination and imaged first to a variable pinhole and than to the detector (Avalanche Photodiode EG&G SPCM AQ161 or PMT Hamamatsu R5600-03). The labeled CuZnSOD exhibited an autocorrelation time corresponding to a hydrodynamic radius of 41,000 Dalton which is comparable to the expected molecular weight of the homodimer (34,600 Dalton). Next 0.2 μl calcineurin-A/B heterodimer (5 μM) was added to a labeled CuZnSOD mixture and the fluorescence correlation signal was determined. The hydrodynamic radius increases from 41 kDa to 90 kDa indicating that approximatly one calcineurin heterodimer interacts with one CuZnSOD dimer (expected: 114 kDa). Using mutated D90A CuZnSOD yielded an apparent molecular weight of 180.000 kDa indicating the formation of calcineurin/CuZnSOD aggrega-

tes. The apparent binding constant between human wild-type CuZnSOD and calcineurin was estimated as k_D = 2 x 10^{-6} M +/- 1 x 10^{-6} M. It is concluded that laser correlation spectroscopy is useful to perform a ultra high throughput screening for ligands diminishing the CuZnSOD/calcineurin interaction which simply can be monitored by a reduction of the autocorrelation time after addition of a potential drugs. It is possible to screen for suitable substances using substances available in chemical, peptide or natural compound screening libraries.

22. High Throughput BioAssay using recombinant calcineurin-A, recombinant calcineurin-B, calmodulin and RII-Fluophos (analytical assay to identify activators or inhibitors of calcineurin) - RII peptide was synthesized according to standard peptide synthesis protocols ((Blumenthal et al., 1988); Interactiva, Ulm, Germany). To generate a fluorescent labeled peptide which furthermore contains a phosphoester at Ser-15, amino acid residue Ser-15 was coupled with fluoresceine-phosphoamidit (FluoreDite Labeling Reagent, Perseptive Biosystems), which is usually used for labeling of nucleotides, to yield RII-Fluophos (Interactiva, Ulm, Germany). The expected molecular weight (2578.8 Dalton) was confirmed by mass spectrometry (2580.6 Dalton). The Fluophos-RII-peptide was not converted by calcineurin as was monitored by fluorescence spectrometrie (Biolumin 960 UV-/VIS/fluorescence microplate reader). Therefore Fluophos-RII-peptide was used in laser fluorescence correlation spectroscopy as described in paragraph 20 exept that λ excitation was 488 nm and λ emission was 520 nm. Furthermore, labeled CuZnSOD was substituted by 10 nM Fluophos-RII-peptide yielding a hydrodynamic radius corresponding to 4 kDa (expected 2.6 kDa). After calcineurin addition the molecular weight increases to 100.000 kDa and a binding

constant of K_d = 0.6 x 10^{-6} M is estimated. Binding constants were comparable between the six calcineurin isoforms/splicevariants. It is concluded that laser correlation spectroscopy is useful to perform a ultra high throughput screening for ligands directly substrate binding to calcineurin by simply monitoring the autocorrelation time after addition of potential drugs. By descriminating the binding properties of potential drugs to the six different heterodimer combinations (calcineurin-A-alpha1/calcineurin-B, calcineurin-A-alpha2/calcineurin-B, calcineurin-A-beta1/calcineurin-B, calcineurin-A-beta2/calcineurin-B, calcineurin-A-gamma1/calcineurin-B, calcineurin-A-gamma2/calcineurin-B) it is possible to identify tissue specific and therefore less toxic calcineurin inhibitors.

It is possible to combine the screening procedures described in paragraph 20 and 21 strategically: substances which are able to inhibit the calcineurin-CuZnSOD interaction (positive hit in paragraph 20) but failed to show an effect in procedure 21 (negative hit) are predominantly positive candidates for the therapeutical use in neurological disorders because a toxic immunosuppressive side effect is less probable. Substances which fail to inhibit the calcineurin-CuZnSOD interaction (negative hit) but show an effect in procedure 21 (positive hit) are predominantly positive candidates for immunosuppression. Substances effective in both procedures are likely to be toxic.

23. Cellular BioAssay using eucaryotic cells transfected with calcineurin-A-EGFP fusionprotein or CuZnSOD-EGFP fusion protein - PC12 cells stably transfected with CuZnSOD and calcineurin isoenzymes serve as a model for monitoring the effects of CuZnSOD or calcineurin overexpression in neuronal cells. CuZnSOD reportedly has been implicated to be involved

in the mediation of hypoxie tolerance, whereas calcineurin overexpression is associated with epileptogenesis, Parkinsonism or Alzheimers disease. It is possible to use theses cells subsequently to the identification of potential drugs in screening protocols 20 und 21. Toxicity of potential neuroprotective drugs and the effect on the subcellular distribution of calcineurin-isoforms or CuZnSOD, respectively can be monitored.

24. Pull-Down-BioAssay using histidine tagged recombinant CuZnSOD to purify CuZnSOD interacting ligands (Preparative assay to isolate activators or inhibitors of CuZnSOD/calcineurin interaction from biological sources) - Recombinant purified histidine tagged CuZnSOD in 50 mM sodiumphosphate buffer pH 8.0 was attached to CuZn-NTA magnetic agarose beads by incubating 100 µl beads suspension with 100 µl CuZnSOD solution (0.3 µg/µl) in 96 well microplates for 30 minutes at room temperature on a microplate shaker (600 rpm). CuZn-NTA magnetic beads were generated from Ni-NTA beads by applying the same procedure as described under paragraph 5 (Ni-NTA magnetic agarose beads, Qiagen, Hilden, Germany). The microplate was placed on the 96 well magnet for 1 minute and the supernatant removed from the wells.

Cytosolic ligands were isolated as follows: 200 µl interaction buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole pH 8.0, 0.1 % Tween-80) were added to the CuZn-NTA agarose beads/CuZnSOD containig wells and placed on the 96 well magnet to remove interaction buffer. 100 mg tissue, cells or other biological specimen to be analyzed for CuZnSOD interacting ligands were homogenized in 200 µl lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole pH 8.0, 0.1 % Tween-80) using a dounce homogenizer. The lysate was cleared by 30 min centrifugation at 10,000 g at 4 ºC. The supernatant was

applied to the wells containing CuZn-NTA absorbed recombinant human CuZnSOD, mixed and incubated for 60 minutes at 0 ºC. The microplate was placed on the 96 well magnet for 1 minute to remove the supernatant. After removal of the lysate the wells were washed twice by adding 200 µl interaction buffer. Elution of CuZnSOD and interacting ligands was achieved by addition of 100 µl elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole pH 8.0, 0.1 % Tween-80).

Membranous ligands were isolated as follows: denaturing interaction buffer (6 M guanidine-HCL, 100 mM NaH_2PO_4 pH 8.0, 0.1 % Tween-80) was added to the CuZn-NTA agarose beads/ CuZnSOD containig wells and placed on the 96 well magnet to remove interaction buffer. The pellet from the procedure above was solubilized in 200 µl denaturing interaction buffer (6 M guanidine-HCL, 100 mM NaH_2PO_4 pH 8.0, 0.1 % Tween-80) for 60 minutes at room temperature. The solubilisate was cleared by 30 min centrifugation at 10,000 g at room temperature. The supernatant was applied to the wells containing CuZn-NTA absorbed recombinant human CuZnSOD, mixed and incubated for 60 minutes at room temperature. The microplate was placed on the 96 well magnet for 1 minute to remove the supernatant. The wells were washed once with 200 µl denaturing interaction buffer (6 M guanidine-HCL, 100 mM NaH_2PO_4 pH 8.0, 0.1 % Tween-80) and a second time with 200 µl denaturing wash buffer (8 M Urea, 100 mM NaH2PO4 pH 8.0, 0.1 % Tween--80). Elution of CuZnSOD and interacting ligands was achieved by addition of 100 µl denaturing elution buffer (8 M Urea, 100 mM NaH_2PO_4 pH 4.0, 0.1 % Tween-80).

To remove low molecular weight ligands for HPLC analysis, the eluates (cytosolic or membranous) were ultrafiltered through a 5 kDa membrane as described under 7. Low molecular weight ligands were separated on a preparative reverse phase HPLC

(UV detection at 200 nm). Homogeneity and molecular weight of UV detectable fractions were analyzed by mass spectrometry. High molecular weight ligands (ultrafiltration remainder) were separated on a 10% polyacrylamide gel and protein bands identified by sequencing or MALDI mass spectrometrie as described under 19. Interacting nucleic acid was analyzed by separating the membranous eluate on a 1 % agarose gel and staining with ethidium bromide. Fluorescent bands were extracted from the agarose (Qiagen gel extraktion kit, Qiagen, Hilden, Germany) subjected to digestion with RsaI and subcloned into RsaI treated pQE30 vector for DNA sequencing.

25. Pull-Down-BioAssay using histidine tagged recombinant calcineurin-A and calcineurin-B to purify calcineurin interacting ligands (Preparative assay to isolate activators or inhibitors of CuZnSOD/calcineurin interaction from biological sources) - Isolation and identification of calcineurin interacting ligands was performed analogous to paragraph 24 with the exception that recombinant calcineurin-A/B heterodimer was attached to Fe-NTA magnetic agarose beads which were prepared as described under paragraph 15. Furthermore six different heterodimer combinations were used (calcineurin-A-alpha1/calcineurin-B, calcineurin-A-alpha2/calcineurin-B, calcineurin-A-beta1/calcineurin-B, calcineurin-A-beta2/calcineurin-B, calcineurin-A-gamma1/calcineurin-B, calcineurin-A-gamma2/calcineurin-B) to descriminate between isoenzyme and splicevariant specific interaction partners.

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Content of sequence listing

1. eukaryotic expression vector CuZnSOD-EGFP (CuZnSOD-pEGFP) (DNA)
2. CuZnSOD (PRT)
3. EGFP (PRT)
4. eukaryotic expression vector EGFP-Calcineurin A alpha (CNAa-pEGFP) (DNA)
5. EGFP (PRT)
6. Calcineurin A alpha (PRT)
7. eukaryotic expression vector EGFP-Calcineurin A beta (CNAb-pEGFP) (DNA)
8. EGFP (PRT)
9. Calcineurin A beta (PRT)
10. eukaryotic expression vector EGFP-Calcineurin A gamma (CNAg-pEGFP) (DNA)
11. EGFP (PRT)
12. Calcineurin A gamma (PRT)

13. prokaryotic expression vector His-CuZnSOD (CuZnSOD-pQE30) (DNA)
14. CuZnSOD (PRT)
15. prokaryotic expression vector CuZnSOD-His (CuZnSOD-pQE60) (DNA)
16. CuZnSOD (PRT)
17. prokaryotic expression vector His-Calcineurin A alpha1-Calcineurin B (CNAa1-pQE30) (DNA)
18. Calcineurin A alpha1 (PRT)
19. Calcineurin B (PRT)
20. prokaryotic expression vector His-Calcineurin A alpha2-Calcineurin B (CNAa2-pQE30) (DNA)
21. Calcineurin A alpha2 (PRT)
22. Calcineurin B (PRT)
23. prokaryotic expression vector His-Calcineurin A beta1-Calcineurin B (CNAb1-pQE30) (DNA)
24. Calcineurin A beta1 (PRT)
25. Calcineurin B (PRT)
26. prokaryotic expression vector His-Calcineurin A beta2-Calcineurin B (CNAb2-pQE30) (DNA)
27. Calcineurin A beta2 (PRT)
28. Calcineurin B (PRT)
29. prokaryotic expression vector His-Calcineurin A gamma1-Calcineurin B (CNAg1-pQE30) (DNA)
30. Calcineurin A gamma1 (PRT)
31. Calcineurin B (PRT)
32. prokaryotic expression vector His-Calcineurin A gamma2-Calcineurin B (CNAg2-pQE30) (DNA)
33. Calcineurin A gamma2 (PRT)
34. Calcineurin B (PRT)
35. peptide RII (PRT)